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BRIEF COMMUNICATION

Activities of ribulose-1,5-bisphosphate carboxylase/oxygenase and phospho*enol*pyruvate carboxylase, and oxygen evolution in transgenic tobacco plants

J. KVĚTOŇ

Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, and Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

Abstract

Three clones of tobacco transformed with the T-DNA of *Agrobacterium rhizogenes* Ri plasmid A4b cultivated *in vitro* on a solid agar medium neither showed pronounced morphological diversity nor significantly differed in chlorophyll (Chl) contents from control plants. However, the transformation caused a 27 to 83 % decay in leaf oxygen evolution and in both ribulose-1,5-bisphosphate carboxylase (RuBPC) and phospho*enol*pyruvate carboxylase (PEPC) activities. Therefore, the transformation brought about a reduction of active PEPC as well as activable RuBPC amounts in plant tissues. Individual clones did not mutually differ. In tobacco transformed with *A. rhizogenes* strain TR101 and grown in soil only the mean leaf area tended to reduce. Chl contents, Chl *a*/*b* ratio, oxygen evolution, and activities of both RuBPC and PEPC were insignificantly affected by the transformation.

Additional key words: *Agrobacterium rhizogenes*, *Nicotiana tabacum*, Ri plasmids, T-DNA, young, mature and old leaves.

T-DNA of *Agrobacterium rhizogenes* Ri plasmids A4b bears genes for agropine synthesis and utilisation (Petit *et al.* 1983) and consists of two regions, T_L and T_R, that integrate independently into a plant genome. The T_R has genes for auxin synthesis (Offringa *et al.* 1986). The T_L contains *rolA, rolB, rolC*, and *rolD* genes the expression of which may not only affect the metabolism of phytohormones but also sensitise plant cells to phytohormone action (*e.g.*, Estruch *et al.* 1991, Dehio *et al.* 1993, Nilsson *et al.* 1993, Schmülling *et al.* 1993). These genes in integration bring about the formation and growth of roots. The Ri plasmid TR101 is of a mannopine type and has a single T-DNA part homologous to T_L of A4b (Filetici *et al.* 1987). Therefore the transformation of plants with Ri plasmids may cause complex physiological modifications by shifting the balance of phytohormone amounts and their activities. The introduction of T-DNA genes from the Ri plasmid A4b into the tobacco genome did not cause statistically significant differences in many structural and physiological traits of the plants (Šiffel *et al.* 1988, Tichá *et al.* 1988). The transformation only broadened their variability range. In comparison with control plants, a higher transpiration rate but still a constant total net photosynthetic rate were found in tobacco clones of the T-phenotype transformed with T-DNA of the Ri plasmid TR101 and transplanted into *ex vitro* conditions (Václavík *et al.* 1988).

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Abbreviations: ATP - adenosine 5'-triphosphate; Chl - chlorophyll; NADH - β-nicotinamide adenine dinucleotide, reduced; PEPC - phospho*enol*pyruvate carboxylase; Ri - root inducing; RuBPC - carboxylating operation of ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; Tricine - N-tris[hydroxymethyl]methylglycine.

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Fax: (+420) 38 5300356, e-mail: queton@umbr.cas.cz

The objectives of this study were to find whether the introduction of Ri plasmid T-DNA genes into the tobacco genome induces some changes in the photoautotrophic carboxylation mediated by RuBPCO), in the non-photo autotrophic carboxylation by PEPC, in oxygen evolution by leaf pieces, in Chl contents, and in leaf area. I studied both the original transformants *in vitro* (A4b) and groups of differently old leaves of the transformants transferred into soil (TR101).

Control and transgenic tobacco (*Nicotiana tabacum* L. cv. White Burley) regenerants transformed with *A. rhizogenes* Ri plasmid A4b or TR101 were kindly provided by Dr. M. Ondřej (IPMB, ASCR, České Budějovice, Czech Republic). Methods of transformation, selection of transgenic plants and their cultivation *in vitro* on the Murashige and Skoog medium without sucrose are described in Tichá *et al.* (1988) and Václavík *et al.* (1988). Plants transformed with the Ri plasmid TR101 were transferred into pots with soil and grown in a greenhouse (Václavík *et al.* 1988). All the plant types were taken for analysis about 40 d after their last transplantation.

Ten to twenty leaf discs (5 - 10 cm^2) cut from a leaf were extracted by a *Potter & Elvejhem* all glass homogenizer (*Kavalier*, Sázava, Czech Republic) in 5 cm^3 of an ice-cold medium containing 50 mM Tricine, 10 mM $MgCl_2$, 0.15 mM 2-mercaptoethanol, 10 mg cm^{-3} polyvinylpyrrolidone, 0.5 mM ethylenediaminetetraacetic acid, at pH 8.0 (KOH). The crude homogenate was filtered through paper filters 595½ and 602½ (*Schleicher & Schuell*, Feldbach, Switzerland). The filtrate was kept in crushed ice until assayed. Enzyme activities were determined at 30 °C in a final volume of 1 cm^3 by continuous monitoring co-factor (NADH) oxidation at 340 nm using a double beam spectrophotometer (*PU 8800, Philips - Pye Unicam*, Cambridge, UK). The RuBPC activity assay was based on the method of Lilley and Walker (1974). The reaction mixture contained: 50 mM Tricine and 20 mM $MgCl_2$ at pH 8.0 (KOH), 5 mM ATP, 5 mM phosphocreatine, 0.2 mM NADH, 42 nkat cm^{-3} glyceraldehyde phosphate dehydrogenase, 67 nkat cm^{-3} phosphoglycerate phospho-kinase, 17 nkat cm^{-3} creatine phosphokinase, 0.5 mM ribose-5-phosphate, 10 mM $NaHCO_3$, and 50 mm^3 of the enzyme extract. The PEPC activity was measured using the conventional coupled enzyme assay (Lane *et al.* 1969). The medium contained: 50 mM Tricine and 5 mM $MgCl_2$ at pH 8.0 (KOH), 0.2 mM NADH, 37 nkat cm^{-3} malate dehydrogenase, 1 mM phospo*enol*pyruvate, 5 mM $NaHCO_3$, and 50 mm^3 of the extract. For measurement of oxygen evolution, leaf discs (2.5 - 5.0 cm^2) were cut by a keen blade into 1 - 2 mm^2 pieces and infiltrated with distilled water for 1 min using a vacuum pump. When exposed to continuous light in a chamber with the reaction mixture (50 mM Hepes, 330 mM mannitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediaminetetraacetic acid disodium salt, and 20 mM $NaHCO_3$), the leaf pieces produced oxygen that was measured by the Clark-type probe: Chl amounts were estimated spectrophotometrically in 80 % (v/v) acetone extracts by the double-wave method (Arnon 1949). Statistical significance of differences between transgenic and control plants was analysed using Mann-Whitney's non-parametric test (*SOLO* software, release *3.1*).

Three independent lines denoted A4b-1, A4b-2, and A4b-17 of the transgenic tobacco regenerants transformed with Ri plasmid A4b T-DNA and cultivated *in vitro* on a solid agar medium were randomly selected for experiments. In the variants A4b-1 and A4b-2, Tichá *et al.* (1988) found insignificant changes in morphological, anatomical, and growth parameters. Similarly in this study, the transformants of all the three clones differed neither in morphology, Chl contents nor Chl *a/b* ratio from the control plants (Fig. 1). On the other hand, the oxygen evolution rate and the activities of bothRuBPC and PEPC (normalized per unit of Chl and/or

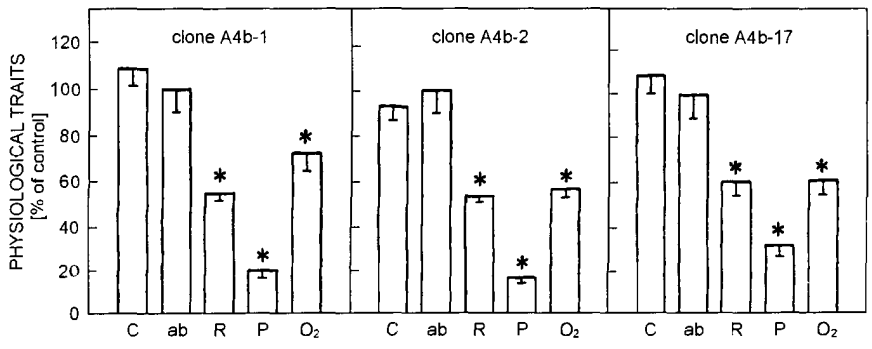

Fig. 1. The effect of transformation with T-DNA of *A. rhizogenes* Ri plasmids A4b on some physiological traits of tobacco. Values in transformants are expressed as percentages of respective controls. C - chlorophyll *a+b* contents, ab - chlorophyll *a/b* ratio, R - RuBPC activity, P - PEPC activity, O_2 - oxygen evolution. *Vertical bars* represent SE from at least 3 independent measurements. *Asterisks* indicate statistically significant differences between transformants and controls at 5 % probability level.

leaf area) were 27 to 83 % lower in transgenic plants than in controls (Fig. 1). However, the individual clones of transformants did not mutually differ. The constant Chl contents and the unchanged Chl *a/b* ratio indicated no effect of the transformation on pigment composition. But the transformation caused a depression of the quantum yield of oxygen evolution and also a reduction of the activable RuBPCO. As CO_2 exchange rate and the growth did not significantly differ between the transgenic clones and the controls (Tichá *et al.* 1988), the decrease in active RuBPCO due to the transformation could imply a higher activation state of this enzyme *in vivo* under the conditions of plantlet cultivation at low irradiance (~ 40 μmol m^{-2} s^{-1}). RuBPC/PEPC activity ratio that increased 1.9 to 3.2 times in transgenic plants (Table 1) proves a larger relative drop of the non-autotrophic CO_2 fixation (through PEPC) in comparison with the autotrophic one. Plantlets were cultivated in vessels covered with Al-foils which allowed merely a low gas exchange. This situation deprives plantlets of external CO_2 and its level drops close to the compensation concentration shortly after the beginning of photoperiod (Pospíšilová *et al.* 1989, Šantrůček *et al.* 1991). Such conditions of growth affect carbon metabolism in plant tissues. Sallanon *et al.* (1995) found a more than 100 % increase of RuBPC activity in walnut plantlets grown mixotrophically at low irradiance in vessels flushed with air containing atmospheric CO_2 concentration (360 cm^3 m^{-3}) compared to those in quasi confined vessels (lower CO_2 concentration); PEPC activity remained unchanged and the RuBPC/PEPC activity ratio was then enhanced. RuBPC activity was higher for *in vitro* autotrophic plantlets of *Rosa hybrida* aerated with ambient CO_2 concentration and/or CO_2-enriched air (2 500 cm^3 m^{-3}) than for control in closed vessels. PEPC activity was stimulated only in the latter case (Genoud-Gourichon *et al.* 1996). Comparison of my results with the findings of these authors suggest that both the transgenic and control tobacco plantlets closed in cultivation flasks and suffering from the shortage of CO_2 during the light period might be endowed with altered photosynthetic (RuBPC) as well as non-photosynthetic (PEPC) CO_2 assimilation.

Table 1. Ratio of RuBPC/PEPC activities in transgenic tobacco transformed with Ri plasmids A4b or TR101. Means ± SE from minimum 5 replications.

Transformant A4b	RuBPC/PEPC	Transformant TR101		RuBPC/PEPC
control	2.9 ± 0.3	young leaves	control	2.0 ± 0.2
clone A4b-1	8.0 ± 1.1		TR101	1.9 ± 0.5
clone A4b-2	9.2 ± 0.9	mature leaves	control	4.3 ± 1.9
clone A4b-17	5.4 ± 0.8		TR101	3.2 ± 0.5
		old leaves	control	4.0 ± 1.0
			TR101	3.2 ± 0.6

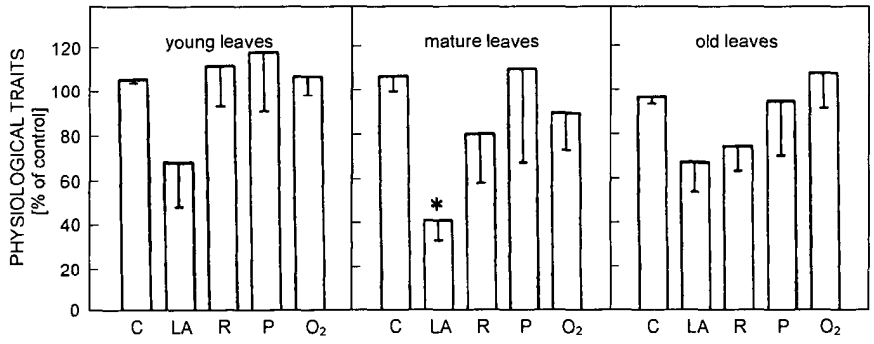

Fig. 2. The effect of transformation with T-DNA of *A. rhizogenes* Ri plasmids TR101 on some physiological traits of tobacco. Values in transformants are expressed as percentage of respective controls. C - chlorophyll *a+b* contents, LA - mean leaf area, R - RuBPC activity, P - PEPC activity, O_2 - oxygen evolution. Statistical treatment is identical to that given in Fig. 1.

The tobacco transformants TR101 did not significantly differ from the controls in the amounts of Chl *a* and *b* and their ratio, in the oxygen evolution by leaf pieces, and in the RuBPC and PEPC activities (on a Chl and/or leaf area basis) in any leaf age class examined (Fig. 2). The RuBPC/PEPC activity ratio was insignificantly different between the transgenic and control plants and tended to a standard dependence on the

leaf insertion level in both plantlet types (Table 1). The mean leaf area was lower in all age categories but the differences were significant only for mature leaves (Fig. 2).

Physiological changes brought about by some transformation depend to a certain extent on the conditions of plant cultivation (Ooms *et al.* 1986). Some regulation mechanisms could lead to the normalization of plant gene expressions in the *ex vitro* regenerants grown in soil under standard environment. Moreover, the expression of transgenes decreases with time (Binns 1983). Hence during the relatively long period, which elapsed from the time of transformation to the realization of experiments, the activity of introduced genes could be gradually suppressed. Mannopine synthesis was used by Václavík *et al.* (1988) as a measure of expression of TR101 T-DNA genes in transgenic tobacco clones of the same origin. The authors indeed demonstrated a slowly decreasing synthesis of mannopine during the development of plants and also showed that the growth parameters tended to normalize as the plants grew.

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Binns, A.N.: Host and T-DNA determinants of cytokinin autonomy in tobacco cells transformed by *Agrobacterium tumefaciens*. - Planta **158**: 272-279, 1983.

Dehio, C., Grossmann, K., Schell, J., Schmülling, T.: Phenotype and hormonal status of transgenic tobacco plants overexpressing the *rolA* gene of *Agrobacterium rhizogenes* T-DNA. - Plant mol. Biol. **23**: 1199-1210, 1993.

Estruch, J.J., Schell, J., Spena, A.: The protein encoded by the *rolB* plant oncogene hydrolyses indole glucosides. - EMBO J. **10**: 3125-3128, 1991.

Filetici, P., Spanò, L., Costantino, P.: Conserved regions in the T-DNA of different *Agrobacterium rhizogenes* root inducing plasmids. - Plant mol. Biol. **9**: 19-26, 1987.

Genoud-Gourichon, C., Sallanon, H., Coudret, A.: Effects of sucrose, agar, irradiance and CO_2 concentration during the routing phase on the acclimation of *Rosa hybrida* plantlets to *ex vitro* conditions. - Photosynthetica **32**: 263-270, 1996.

Lane, D., Maruyama, H., Easterday, R.L.: Phosphoenolpyruvate carboxylase from peanut cotyledons. - In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 13. Pp. 277-283. Academic Press, New York - London 1969.

Lilley, R.M.C., Walker, D.A.: An improved spectrophotometric assay for ribulosebisphosphate carboxylase. - Biochim. biophys. Acta **358**: 226-229, 1974.

Nilsson, O., Moritz, T., Imbault, N., Sandberg, G., Olsson, O.: Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* T_L-DNA. - Plant Physiol. **102**: 363-371, 1993.

Offringa, I.A., Melchers, L.S., Regensburg-Tuink, A.J.G., Costantino, P., Schilperoort, R.A., Hooykaas, P.J.J.: Complementation of *Agrobacterium tumefaciens* tumor-inducing *aux* mutants by genes from the T_R-region of the Ri plasmid of *Agrobacterium rhizogenes*. - Proc. nat. Acad. Sci. USA **83**: 6935-6939, 1986.

Ooms, G., Atkinson, J., Bossen, M.E., Leigh, R.A.: T_L-DNA from *Agrobacterium rhizogenes* plasmid pRi1855 reduces the osmotic pressure in transformed plants grown *in vitro*. - Planta **168**: 106-112, 1986.

Petit, A., David, C., Dahl, G.A., Ellis, J.G., Guyon, P., Case-Delbart, F., Tempé, J.: Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. - Mol. gen. Genet. **190**: 204-214, 1983.

Pospíšilová, J., Tichá, I., Čatský, J., Solárová, J., Ondřej, M.: Photosynthesis in regenerants of tobacco transformed by plasmids of *Agrobacterium*. 3. Transplanted plants. - Photosynthetica **23**: 130-135, 1989.

Sallanon, H., Dimon, B., Carrier, P., Chagvardieff, P.: Effects of CO_2 concentration and irradiance on growth and photosynthesis of *Juglans regia* plantlets grown *in vitro*. - Photosynthetica **31**: 241-249, 1995.

Šantrůček, J., Šiffel, P., Polišenská, M., Pešková, H.: Species-specific response of the photosynthetic apparatus of tobacco and potato to low CO_2 concentrations. - Photosynthetica **25**: 203-209, 1991.

Schmülling, T., Fladung, M., Grossmann, K., Schell, J.: Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. - Plant J. **3**: 371-382, 1993.

Šiffel, P., Durchan, M., Květoň, J., Ondřej, M., Šesták, Z.: Photosynthesis in regenerants of tobacco transformed by plasmids of *Agrobacterium*. 2. Fluorescence emission spectra and age induced changes in slow fluorescence induction. - Photosynthetica **22**: 214-220, 1988.

Tichá, I., Ondřej, M., Hrouda, M., Čatský, J., Zima, J., Pospíšilová, J., Solárová, J.: Photosynthesis in regenerants of tobacco transformed by plasmids of *Agrobacterium*. I. Morphology, anatomy and gas exchange of transformants. - Photosynthetica **22**: 350-363, 1988.

Václavík, J., Konečná, A., Ondřej, M.: Effect of pRi T-DNA transformation on growth and gas exchange of transplanted tobacco regenerants of the T and T′ phenotypes. - Biol. Plant. **30**: 432-439, 1988.